

**CORRECTION OF GLUCOSE-6-PHOSPHATE
DEHYDROGENASE (G6PD) VIANGCHAN
MUTATION IN MONOCYTES USING
CRISPR/CAS9**

LELAMEKALA A/P VENGIDASAN

**UNIVERSITI SAINS MALAYSIA
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MUTATION IN MONOCYTES USING
CRISPR/CAS9**

by

LELAMEKALA A/P VENGIDASAN

**Thesis submitted in fulfilment of the requirements
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LIST OF SYMBOLS AND ABBREVIATIONS

μ	micro
aa	amino acid
AAV	adeno-associated virus
ALA	aminolevulinic acid
APS	ammonium persulfate
APS	ammonium persulfate
ATP	adenosine triphosphate
bp	base pairs
CO ₂	carbon dioxide
CRISPR	clustered regularly interspaced short palindromic repeats
Da	dalton
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DSB	double stranded break
ECL	enhanced chemiluminescence
<i>E coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
g	gram
G6PD	glucose 6 phosphate dehydrogenase
GOI	gene of interest
H ₂ O ₂	hydrogen peroxide
HDR	homology directed repair
HFV	human foamy virus

HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IPTG	isopropyl β -D-1-thiogalactopyranoside
kDa	kilo dalton
L	litre
m	milli
M	molar
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
min	minutes
mRNA	messenger RNA
n	nano
NADP	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
NHEJ	non-homologous end joining
OD	optical density
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
pH	potential of hydrogen
PK	pyruvate kinase
PPP	pentose phosphate pathway
PRSA	pure red cell aplasia
RNA	ribonucleic acid
ROS	reactive oxygen species

rpm	revolutions per minute
SCID-ADA	adenosine deaminase deficiency
SCID-XI	X-linked severe combined immunodeficiency
SDS	sodium dodecyl sulfate
sec	second
TBST	tris-buffered saline
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
TRIS	tris (hydroxymethyl) aminomethane

**PEMBETULAN MUTASI GLUCOSE-6-PHOSPHATE DEHYDROGENASE
(G6PD) VIANGCHAN DALAM MONOSIT DENGAN MENGGUNAKAN
CRISPR/CAS9**

ABSTRAK

Kekurangan G6PD adalah antara penyakit enzim yang paling biasa dihadapi manusia dengan kira-kira 400 juta orang yang terjejas olehnya di seluruh dunia. Dua ratus tujuh belas jenis mutasi telah ditemui setakat ini dan ada sebilangan jenis mutasi ini boleh menyebabkan maut. Kekurangan G6PD juga dikaitkan dengan pelbagai jenis kanser, tumor dan penyakit metabolik. Kajian ini bertujuan untuk membaiki kekurangan G6PD dengan menggunakan sistem CRISPR / Cas9. Kajian ini menggunakan G6PD Viangchan, sejenis mutasi yang lazim dijumpai dalam kalangan etnik Melayu Malaysia. G6PD Viangchan telah diklon ke dalam plasmid pengekspresan pET26b (+) dan diekpreskan di dalam sistem BL21 (DE3). Protein rekombinan G6PD Viangchan yang terhasil telah disahkan dengan membandingkan aktiviti enzimnya dengan G6PD *wildtype*. Keputusan menunjukkan protein G6PD Viangchan telah berjaya dihasilkan dengan menggunakan sistem ekspresi heterologous. Ekspresi enzim telah dikurangkan sehingga 67% berbanding G6PD *wildtype*. Seterusnya, G6PD Viangchan telah diklonkan ke dalam plasmid pengekspresan lentiviral, pLJM-eGFP, untuk menghasilkan zarah lentiviral. Virus ini digunakan untuk mentransdusi sel THP-1 untuk menghasilkan sel THP-1 yang mengekpres protien G6PD Viangchan secara stabil. Enzim yang dihasilkan oleh sel tersebut telah disahkan dengan menggunakan ujian aktiviti enzim. Keputusan menunjukkan sel THP-1/G6PD Viangchan menghasilkan tahap enzim G6PD yang lebih rendah (lebih kurang 50 %) berbanding dengan sel THP-1 yang tidak

ditransduksi. Akhirnya, kekurangan tersebut telah diperbaiki dengan menggunakan sistem suntingan gen CRISPR / Cas9. *GuideRNA* (gRNA) yang mengapit kawasan mutasi telah dihasilkan menggunakan kaedah PCR dan telah ditranskripkan kepada RNA. Sel THP-1/G6PD Viangchan telah ditransfek dengan menggunakan gRNA, DNA Cas9 dan DNA donor dengan menggunakan Lipofectamine 3000. Walaupun begitu, suntingan gen berasaskan CRISPR hanya berupaya meningkatkan sebanyak 8.8 % enzim G6PD berbanding sel kawalan yang tidak ditransdusi. Pengoptimuman untuk suntingan gen berasaskan CRISPR amat diperlukan untuk pembaikan gen yang lebih tinggi. Meskipun begitu, kajian ini membentuk satu struktur asas dalam menyediakan pendekatan baru untuk terapi kekurangan enzim G6PD dengan menggunakan pendekatan molekular.

**CORRECTION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE
(G6PD) VIANGCHAN MUTATION IN MONOCYTES USING
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ABSTRACT

G6PD deficiency is one of the most common enzymopathy in human with approximately 400 million people affected worldwide. Two hundred seventeen types of mutations have been described so far with some mutations are life threatening. G6PD deficiency has also been linked to various types of cancers, tumours and metabolic diseases. This study aimed to correct G6PD deficiency by using CRISPR/Cas9 system. In this study, G6PD Viangchan as used; a type of mutation which is common in Malaysian Malays. G6PD Viangchan has cloned into pET26b (+) expression plasmid and expressed in BL21 (DE3) system. The recombinant G6PD Viangchan protein was verified by comparing its enzyme activity with G6PD wild type. Results showed a successful production of G6PD Viangchan protein by using heterologous expression system. The enzyme expression was reduced up to 67 % compared to G6PD WT. Subsequently, G6PD Viangchan was cloned into a lentiviral expression plasmid, pLJM-eGFP, to produce lentiviral particles. These particles were used to transduce THP-1 cell line, in order to generate stable G6PD Viangchan-expressing cells. The stable cell line was verified by using enzyme activity assay. THP-1/G6PD Viangchan cells produced a lower amount of G6PD enzyme (approximately 50 %) compared to untransduced THP-1 cells. Consecutively, the deficient was corrected by using CRISPR/Cas9 genome editing system. Guide RNAs flanking the mutation site were produced by using PCR approach and *in vitro* transcribed into RNA. G6PD deficient THP-1 cells were transfected with gRNA, Cas9 DNA and donor DNA by

using Lipofectamine 3000. However, CRISPR-based gene editing only managed to increase approximately 8.8 % of G6PD enzyme compared to the untransfected control cells. Optimisations for CRISPR-based gene editing are much needed to obtain a higher percentage of gene correction. Nevertheless, this study forms a fundamental structure in providing a new opportunity in therapy for G6PD deficiency by using molecular approach.

1.0 GENERAL INTRODUCTION

Red blood cells (RBC) require a source of energy to efficiently perform its function and for its survival in the circulation for approximately 120 days (Brouillard, 1974). Glucose is the normal energy source of the red blood cell which is metabolised by the RBC along two major routes, the glycolytic pathway and the pentose phosphate pathway (PPP) (Xiao et al., 2018). In PPP pathway, glucose-6-phosphate is oxidized to 6-phosphoglucono- δ -lactone. The reaction is catalysed by glucose-6-phosphate dehydrogenase (G6PD) enzyme and coupled with NADP⁺ reduction into NADPH (Patra and Hay, 2014). NADPH is used in reducing GSSG to GSH. Normal cells can defend themselves against oxidative stress reducing GSSG to GSH. However, G6PD deficient red cells are unable to reduce NADH to NADPH at the normal rate, hence lowering the conversion rate of hydrogen peroxide or the mixed disulphides into haemoglobin and glutathione (GSH). This metabolic even lead to red cell damage and eventually destruction or haemolysis as discussed in Figure 1.1 (Beutler, 2018).

G6PD deficiency is an X-linked genetic disease which can be inherited. Individuals with G6PD deficiency often appears asymptomatic unless triggered by infection, drugs, mothballs and fava beans (Cappellini and Fiorelli, 2008). These triggers cause RBC to break down prematurely and results in early destruction of red blood cells is called hemolysis (Shalev et al., 1985). Figure 1.2 shows a sketch by Jorge Muniz was published on Twitter in the 7th Sep 2015 on the condition of G6PD deficiency. Sketch was upload to create more awareness about the disease among the common people. It is shown that the red blood cells undergo series of haemolysis after exposed to oxidative drugs such as aspirin, sulphonamides, nitrofurantoin, dapsone, primaquine and quinidine. Favism and infection can also trigger haemolysis anaemia in the patient.

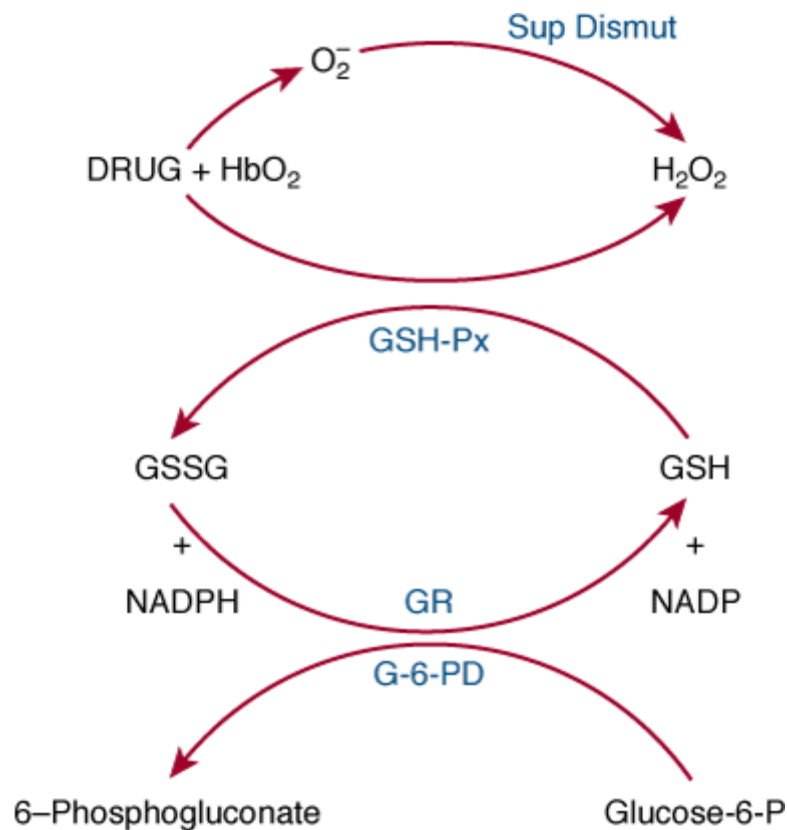


Figure 1.1: Hydrogen peroxide is being generated and detoxified in the erythrocytes. In G6PD patients, insufficient amount of NADPH results in accumulation of GSSH which leads to haemoglobin denaturation and decrease RBC survival. GR-glutathione reductase; GSH Px-glutathione peroxidase; GSSH-glutathione disulphide (oxidized glutathione); Sup Dismut- superoxide dismutase (Williams and Lichtman, 2006).

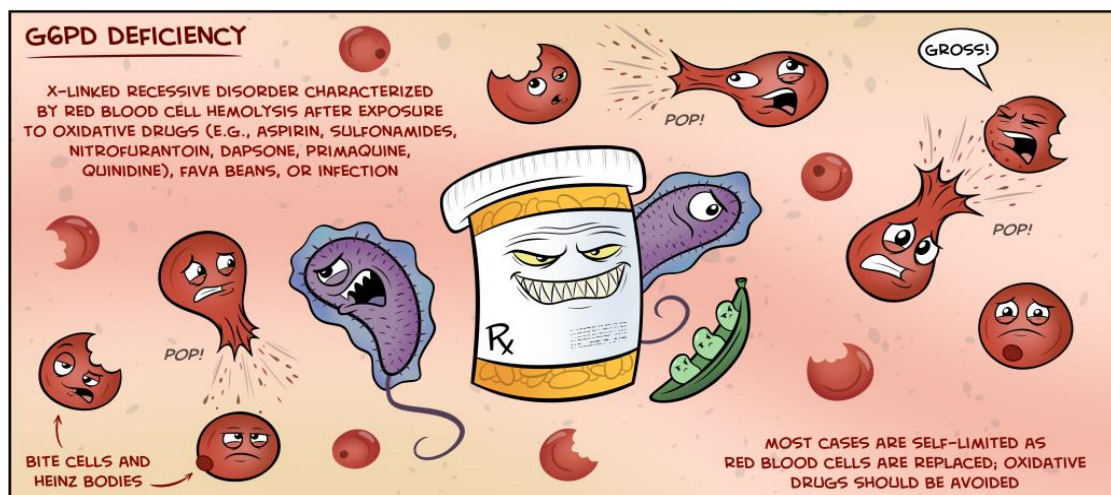


Figure 1.2: Hemolysis in G6PD deficient cells is caused by oxidative drugs, favism and infection. The sketch also illustrates the formation of Heinz body and bite cells due to drug induced hemolysis in G6PD deficient cells (Muniz, 2015).

1.1 Background of problem

G6PD gene is a housekeeping gene present in all cells. G6PD enzyme, a product of G6PD gene is involved in the first enzymatic reaction in pentose phosphate pathway (PPP) by converting glucose-6-phosphate to 6-phosphogluconolactone and simultaneously reduces NADP⁺ to NADPH (Kuby 1962). G6PD is the sole producer of NADPH (Filosa et al., 2003) and plays a significant role to protect cells against oxidative stress and to maintain cells in a reduced state. G6PD deficiency is the most common genetic enzyme deficiencies with approximately 400 million people living with it (Cappellini and Fiorelli, 2008). G6PD deficiency is X-linked genetic disorder affecting men. However, females can also be affected.

G6PD Viangchan variant is classified into G6PD Class II according to World Health Organisation (WHO). The world health organization classified G6PD deficiency variants into five different classes as shown in Table 1.1. G6PD deficiency falls under severe deficiency with less than 10 % of G6PD normal activity and can cause clinical implication such as intermittent haemolysis. G6PD deficiency varies according to its classes. Individuals with G6PD deficiency often suffer from a number of diseases such as neonatal jaundice, favism, drug or infection related haemolytic and chronic non-spherocytic haemolytic anaemia.

G6PD deficiency also has been linked to hepatitis A and E (Hu et al., 2014, Gotsman and Muszkat, 2001, Monga et al., 2003) and dengue infection (Al-alimi et al., 2014, Chao et al., 2008, Tanphaichitr et al., 2002). Additionally, G6PD deficiency is also often associated with high oxidative stress in the cell. High oxidative stress is also linked to cancer. G6PD is found to be activated in many cancers and tumors including breast cancer (Bokun et al., 1986, Pu et al., 2015), cervical cancer (Duțu et al., 1979, Hu et al., 2015), prostate cancer (Zampella et al., 1982, Tsouko et al., 2014),

endometrial cancer (Hughes, 1976), lung tumors (Pisano et al., 1991), renal cancer (Zhang et al., 2017b), ovarian cancer (Yi et al., 2015), bladder cancer (Wang et al., 2015) and gastric cancer (Wang et al., 2012).

Table 1.1: WHO classification for G6PD deficient variants

Types of class	Sereverity	Percentage of activty	Clinical complication
Class I	Severe deficiency	<10 % activity	chronic (nonspherocytic) hemolytic anemia
Class II	Severe deficiency	<10 % activity	intermittent hemolysis
Class III	Mild deficiency	10-60 % activity	hemolysis with stressors only
Class IV	Non-deficient variant	60-150 % of enzyme activity	no clinical sequelae
Class V	Increased enzyme activity	>150 % of normal activity	no clinical sequelae

1.2 Problem statements

G6PD deficiency can be classified into different variants according to the position of this mutation. Viangchan type variant has a mutation that lies on the position 871 on its nucleotide. It is one of the common variant is Malaysian Malay population which comprises of 32.7% followed by Mahidol and Mediterranean variants (Ainoon et al., 2002). Henceforth, the study was designed by targeting the Viangchan type variant. Nevertheless, the prevalence of G6PD deficiency in Malaysia is 3.1 % among males and was shown to be commonest among Malays and Malaysian Chinese and less common among the Indians (Singh, 1986, Hon et al., 1989).

There are no defined treatments for G6PD deficiency. Normally, patients often live by avoiding the G6PD triggers for the entire life. The treatment work more on managing the haemolysis rather than a curing the disease. Patients are often managed by controlling occurrence of haemolytic anaemia that is caused by the triggers. In some severe cases for instance in chronic non-spherocytic haemolytic anaemia (CNSHA), patients might need blood transfusion for their entire life.

1.3 Objectives of the study

General objective:

The main objective of the study is to correct G6PD deficiency in THP-1 cell line by using ribonucleic CRISPR-Cas9 system.

Specific objective:

- 1) To produce full length functional normal and mutated (Viangchan) G6PD protein.
Through this study, protein production and enzyme activity of both wild type and disease protein were studied and compared.
- 2) To generate viral particles by using lentiviral third generation system. Third generation lentiviral system was chosen over the second generation because it produces safer virus particles compared to previous lentiviral system.
- 3) To establish stable G6PD deficient THP-1 cell line by using the produced viral particles. The disease model was expected to produce lower expression of G6PD protein.
- 4) To correct the deficient by using ribonucleic CRISPR/Cas9 system. The correction utilized homology directed repair by using donor template (ssODN) by fixing the single site mutation.

2.0 LITERATURE REVIEWS

2.1 Red blood cells (RBC)

2.1.1 Introduction

The term erythropoiesis was derived from the Greek terms “*erythros*” (red) and “*poiein*” (making) and describes the process of the production of red blood cells (RBC) (Colman, 2009). *Erythron* which include both the progenitor and adult red cells is the term used to emphasise the idea that they function as an organ. RBCs are highly differentiated cells that have no nuclei or cytoplasmic organelles. They do not contain ribosomes; they cannot synthesise protein to replace molecules such as enzymes, structural proteins which become denatured. Normal RBCs are in biconcave disc with an average diameter of 7.2 μm and thick of 2.0 μm as shown in Figure 2.1 (Wickramasinghe & Erber, 2011). RBC have a limited life span of 110-120 days, at the end of which they are ingested and degraded by the macrophages of the marrow, spleen, liver and other organs (Franco, 2012).

2.1.2 Production of red blood cells

Production of RBC or erythropoiesis is a tightly regulated process by which hematopoietic stem cells differentiate into erythroid progenitors and then mature into RBC as shown in Figure 2.2. Erythropoietin (EPO) which is produced in kidneys is the principal hormone regulating erythropoiesis (Jacobson et al., 1957). RBC are produced from multipotent haemopoietic cells (Wickramasinghe, 1975) which reside in the bone marrow and are stimulated by variety of hormones or cytokines. The earliest characterised progenitor committed to the erythroid lineage is the burst forming unit-erythroid (BFU-E). This progenitor requires multiple factors for proliferation and prevention of apoptosis to differentiate into a late progenitor, colony forming unit-erythroid (CFU-E) (Elliott et al., 2008). Beyond the CFU-E stage,

erythroid progenitors are largely regulated by EPO to form erythroblasts, the precursor cells (Lodish et al., 2010). These cells develop from there, proliferate and differentiate into reticulocytes which will be released into the circulation for development into mature RBC.

2.1.3 Function of red blood cells (RBC)

The prime function of the RBC is to combine with oxygen in the lungs and to transport and release this oxygen for utilisation of the tissues. The red cells also combine with carbon dioxide (CO₂) produced in tissues and release this in the lungs. The function of oxygen transport resides in the haemoglobin which is made up of two α (alpha) and two β (beta) subunits as shown in Figure 2.3. To achieve an optimal performance as an oxygen transporter the red cells require a healthy RBC membrane and healthy enzyme systems provide energy and protect against oxidant damage. The RBC membrane is composed of a lipid bilayer and is bound to a sub-membranous cytoskeletal network which is responsible for maintaining the biconcave shape of a normal RBC (Peng et al., 2013).

The membrane contains adenosine triphosphate (ATP)-dependent pumps as shown in Figure 2.4 that counteracting a continuous passive diffusion of ions across the membrane in the opposite direction. In the resting state 90 % of glucose is catabolised anaerobically through the Embden-Meyerhof pathway, which also serves to generate nicotinamide adenine dinucleotide (NAD⁺) in its reduced form NADH, required as cofactor for cytochrome b5 reductase for the conversion of methaemoglobin to haemoglobin.

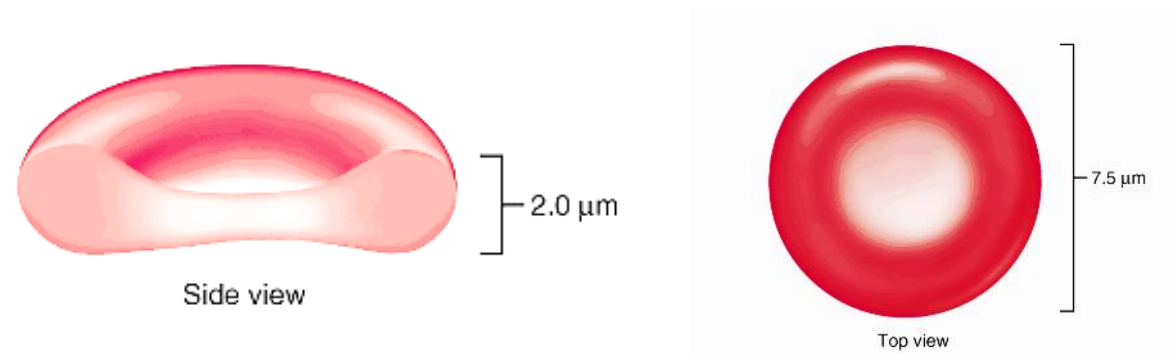


Figure 2.1: Top view and side view of a red blood cell with average diameter of 7.2 μm and average thick of 2.0 μm. It is composed of lipid bilayer and bounded to sub-membranous cytoskeletal network (Marieb and Hoehn, 2007).

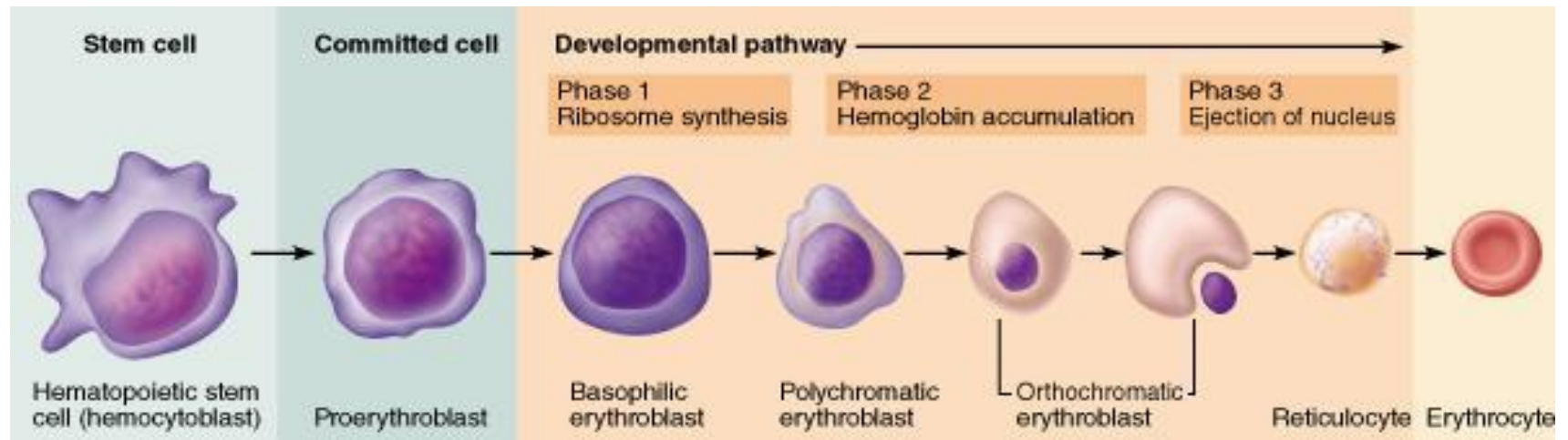


Figure 2.2: Development of RBC from hemocytoblast. Three phases are involved, production of ribosomes, synthesis of haemoglobin and ejection of nucleus and organelles (Marieb and Hoehn, 2007).

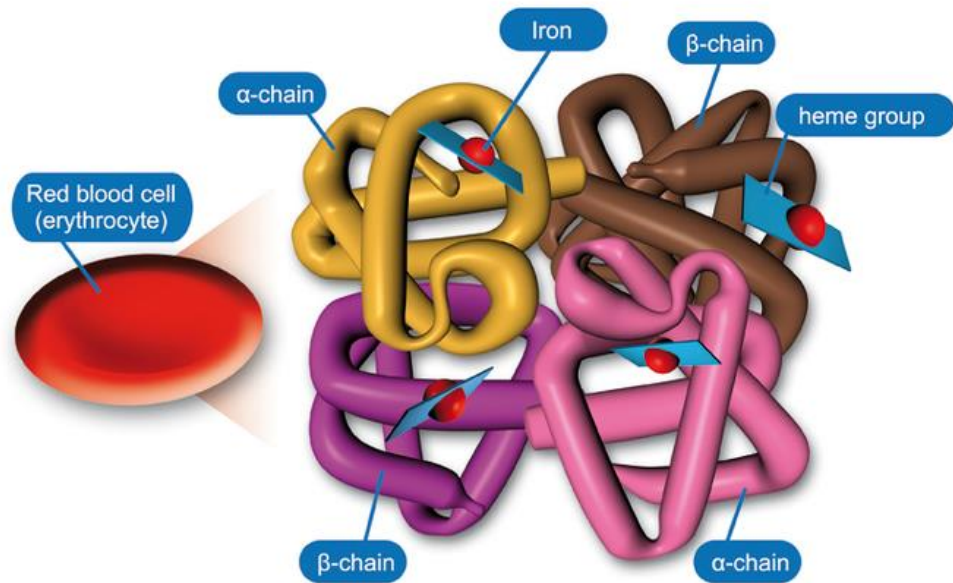


Figure 2.3: Structure of haemoglobin that consist of α and β chains. Each chain consists of a heme group that holds an iron atom respectively (Johnson et al., 1995).

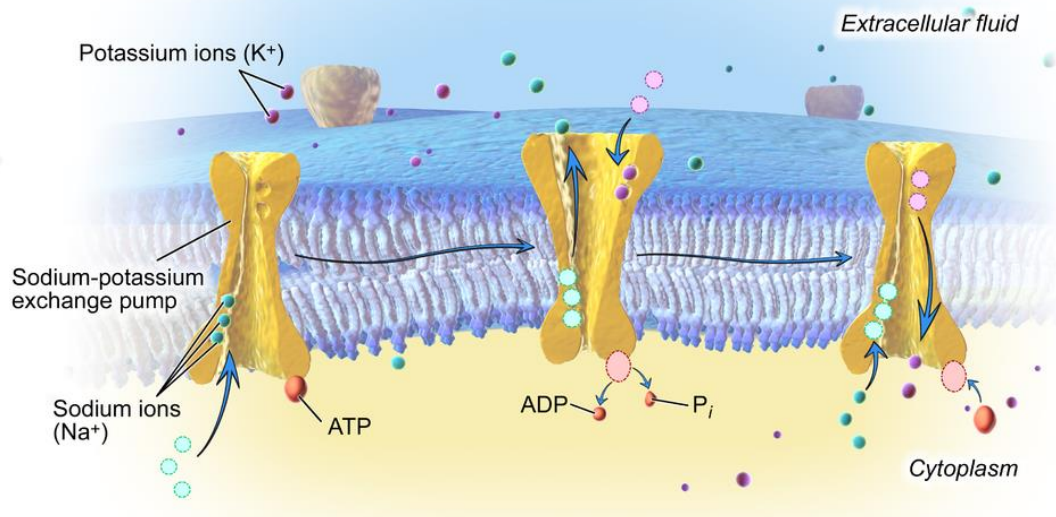


Figure 2.4: The sodium-potassium exchange pump (Staff, 2014). The membrane consists of potassium/sodium ions pump which uses ATP as the source of energy. The top part shows the extracellular fluid and the inner part shows the cytoplasm.

2.2 RBC disorders

Hoffbrand in his book entitled “Postgraduate Haematology” has categorised RBC disorders into numerous subgroups and G6PD deficiency falls under section : Disorders of red blood metabolism (Hoffbrand et al., 2016). Only disorders of red blood metabolism will be discussed in subsequent section.

2.2.1 Disorders of red blood metabolism

RBC play an important role to carry haemoglobin around the blood circulation in optimal concentration and in a functional state to allow an efficient gas exchange in the lungs and tissue capillaries (Beutler and Waalen, 2006). In order to achieve this; the red blood cells need a constant supply of energy in the form of ATP and a source of reducing power (Betz et al., 2009). The energy is derived from anaerobic glycolysis and PPP. ATP is required to maintain the membrane in its deformable state, with asymmetric lipid layers, and to regulate ion and water exchange (Nagy et al., 1997). G6PD enzyme catalyses the first step of PPP which reduces nicotinic adenine dinucleotide phosphate (NADPH) pathway too (Kruger and von Schaewen, 2003). NADPH drives the glutathione cycle, glutathione (GSH) being the major reducing agent within the red cell (Schafer and Buettner, 2001). Reduction of any enzyme in the mature red cells means none of the enzymes in the metabolic pathways can be replaced during the red cell lifespan. Enzyme activities will decline until a new normal red cell is produced.

G6PD enzyme by far is one of the most important enzymes in PPP and its deficiency results in accumulation of reactive oxygen species in cell which will eventually destroy the RBC. G6PD deficiency will be further explained in next section 2.3.

2.3 G6PD DEFICIENCY

2.3.1 History

In the year 1926, a group of individuals developed haemolytic anaemia while being treated for malaria with 6-methoxy-8-aminoquinoline drugs (Beutler et al., 1954). The questions arise whether the drug doesn't compatible to the malaria patient or did the patients' RBC differ in some way. To solve this, Sterling and Gray was first to use ^{51}Cr method to label the RBC and found out that sensitivity of the haemolytic effect of primaquine was due to an intrinsic defect of the RBC (Sterling and Gray, 1950). Few years later, Carson studied the reduction of glutathione by hemolysates. He discovered that hemolysates from primaquine-sensitive men could not utilise glucose-6-phosphatase to reduce glutathione, concluding that primary defect was in glucose-6-P-utilizing enzyme, G6PD (Carson et al., 1956). It was later found that besides primaquine, other drugs including sulphanilamide, acetanilide and some sulfones also trigger haemolysis (Beutler, 2008). Till then, G6PD deficient is still not discovered and drug administration was performed very carefully according to individual's drug ingestion history.

G6PD deficiency was first found in African-American subjects and was restricted to a single ethnic group only. Due to that reason, it was categorised as genetic based disorder (Browne, 1957). The same group also discovered that G6PD deficiency was an X-linked disorder and that affects primarily the RBC, older cells being more severely affected than newly formed. In the year 1961, it become apparent that the disorder did not limited exclusively among people of Africa but also Southern Europe and the Middle East. Besides, Marks and Gross shown that G6PD deficiency in Mediterranean peoples were much more severe than among African Americans. In the

year 1967 (WHO, 1967), World Health Organisation (WHO) published standard method to measure G6PD enzyme and later in 1971 they sponsored a study.

G6PD deficiency classifications according to its enzyme activity was published from the study (Yoshida et al., 1971). By the year 1988, more than 370 variants were described (Beutler and Yoshida, 1988). Since some types of variant found to be redundant, a recent review has re-categorised the different types of variants into 217 types mutation (Gómez-Manzo et al., 2016b, Gómez-Manzo et al., 2017). Although the disorder was discovered in early days, the real mechanism of haemolysis was fully discovered after 30 years. The discovery has opened a lot of door to study biochemical pathways.

2.3.2 Genetics and Inheritance

G6PD gene is located in the distal long arms of the X chromosome at the Xq28 locus (Figure 2.5). The gene contains 13 exons and 12 introns which are over 18.5 kb in length (Martini et al., 1986). G6PD enzymes have 515 amino acids, which translated into 59, 256 Da proteins. G6PD mRNA encodes two isoforms, G6PDH isoform (a) (545 amino acids) and isoform (b) (515 amino acids). The 545 amino acid isoform (a) protein is inactive but post-translational processing results in a 515 amino acid which is the functional protein containing an acetylated alanine residue at the N-terminus.

Biologically active G6PDH is functional as either a homodimer or a homotetramer and both forms co-exist in equal proportions at neutral pH as shown in Figure 2.6. G6PD deficiency is an X-linked recessive disorder. Males usually manifest the abnormality and females are carriers. For this reason, the defect is fully expressed in affected males and is never transmitted from father to son, but only from mother to son. The main cause of the deficiency is single site mutation. Although more than 400 types of mutation has been described so far, a precise molecular characterisation shows

mainly 140 missense mutation leading to amino-acid substitution (Beutler et al., 1996, Mason and Vulliamy, 2003). Despite that, a recent extensive review was published, recognizing 217 mutations in the G6PD gene that are responsible for the severity of the clinical symptoms (Gómez-Manzo et al., 2016b, Gómez-Manzo et al., 2017). Figure 2.7 shows few types of mutations that have been mapped against the G6PD exons.

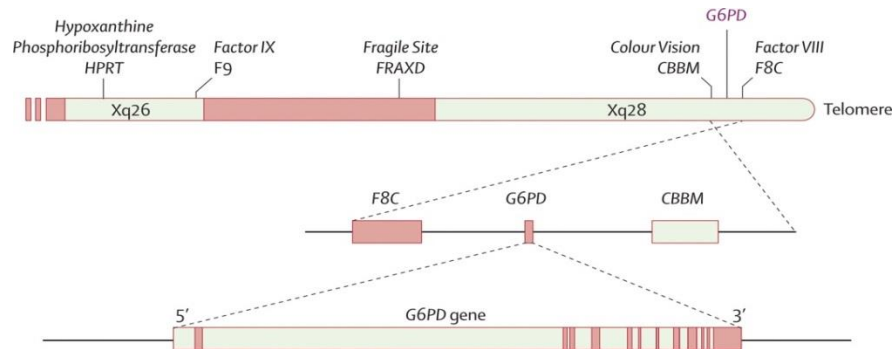


Figure 2.5: Position of G6PD gene in X chromosome. G6PD gene is located at lower arm q at position 28 (Cappellini and Fiorelli, 2008).

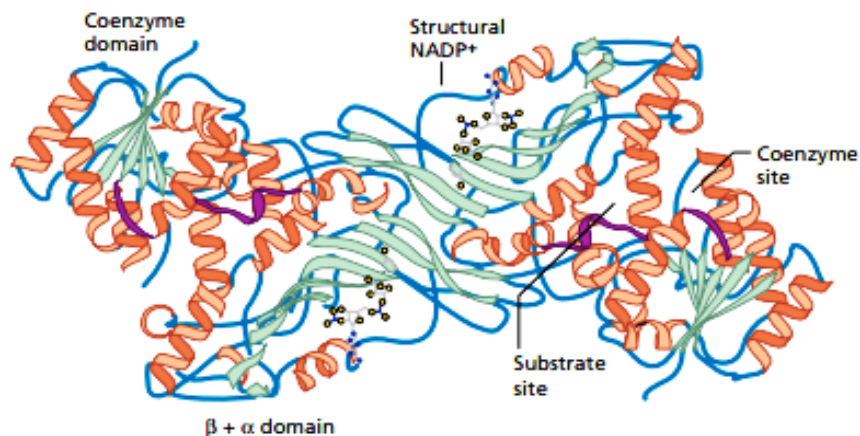


Figure 2.6: Dimer form of G6PD gene structure showing active site for G6P and NADP+ ligand (Hoffbrand et al., 2016).

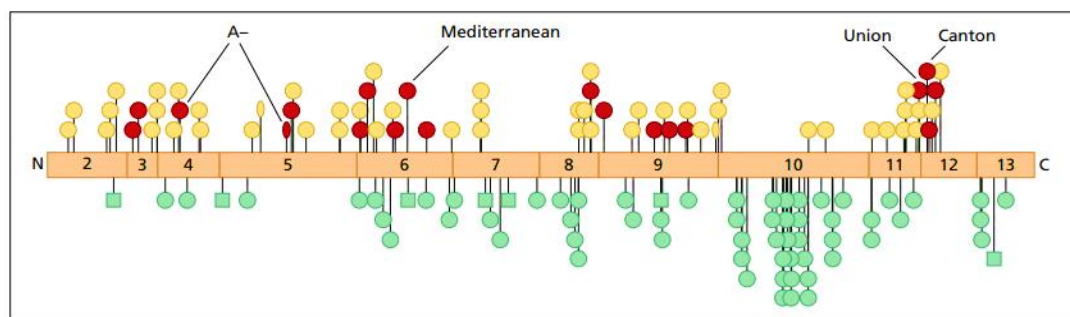


Figure 2.7: G6PD gene map showing mutation sites. Numbers in boxes refer to the location of G6PD exons (courtesy of Tom Vulliamy, Hammersmith Campus, Imperial College, London) (Hoffbrand et al., 2016).

Legends: Numbered boxes refer to the location of G6PD exons: yellow circles, class I and II variants; yellow ellipses, class IV variants; red circles, polymorphic variants; green circles and squares, class I variants caused by amino acid substitutions and small in-frame deletions, respectively.

2.3.3 Pathophysiology

G6PD is expressed in all cells and is responsible for the first enzymatic reaction of PPP in which glucose-6-phosphate is oxidised to 6-phosphogluconolactone with simultaneous production of NADPH as shown in Figure 2.8. The G6PD / NADPH pathway is the sole source of reduced glutathione in RBC (Verrelli et al., 2002, Filosa et al., 2003). The production of NADPH is required for a variety of reductive biosynthetic reactions. One and foremost is for production of reduced form of GSH which plays an important role on detoxification of hydrogen peroxide (H_2O_2) (Gaetani et al., 1996, Scott et al., 1993, Kirkman et al., 1987). H_2O_2 is toxic by-product of cellular metabolism in aerobic organism which is also known as reactive oxygen species (McCord and Fridovich, 1969).

NADPH functions to defend against the oxidizing effects of H_2O_2 (Figure 2.9). The NADPH produced in the PPP, and the antioxidant GSH, are both necessary for the continual removal of ROS from the RBC. When a patient present with G6PD deficiency, he or she is usually asymptomatic unless triggered by oxidant agent known as triggers. The oxidant will creates more free ROS in the red cells. Glutathione predominantly will be active in the cells but without G6PD, they will be failed to act further and stay in their oxidised form. Hence, the free radical forms Heinz bodies and stick to the red cell membrane (Mannoji et al., 1985). Cell membrane damaged by the Heinz bodies and ROS become distorted and the cell is likely to undergo lysis.

2.3.4 Prevalence and Geographic Distribution

The condition affects up to 1 % of the world population but is considerably higher with gene frequencies from 5-25 % in tropical Africa, Middle East, tropical and subtropical Asia, some areas of the Mediterranean and Papua New Guinea as shown in Figure 2.10. The prevalence of G6PD deficiency was found to be higher in malaria endemic countries (Howes et al., 2012). The first identified variant is G6PD A- (G202A/A376G) (Comfort, 2009). The variant is rare for having double point mutation and very common in the sub-Saharan African population. G6PD Mediterranean was predominant in West Asia (from Saudi Arabia and Turkey to India) (Howes et al., 2013). Howes reported multiple variants co-occurring with no single variant being predominant in Southeast Asia (Howes et al., 2013). However the previous report shows Mahidol variant (G487A) is common especially across Myanmar, Thailand and Indonesia (Phompradit et al., 2011).

The prevalence of G6PD deficiency in Malaysia is 3.1 % among males and was shown to be commonest among Malay ethnics and Malaysian Chinese and less common among the Indians. Another small study comprises 87 people shows similar result but a higher prevalence 4.59 % (Sulaiman et al., 2013). The most common type of variant in Malay ethnic is G6PD Viangchan (37.2 %), followed by G6PD Mediterranean (26.7 %) and G6PD Mahidol (15.1 %) (Ainoon et al., 2003, Yusoff et al., 2004). G6PD Viangchan variant carries point mutation at position 871 (G>A).

2.3.5 Viangchan mutation

G6PD Viangchan gets its name from the name of the capital city of Laos which is also known as Vientiane. In the year 1988, G6PD Viangchan was first characterised biochemically from a Laotian immigrant G6PD-deficient patient who lives in Canada (Poon et al., 1988). G6PD Viangchan carries a single nucleotide mutation at position 871 from G to A. The mutation will substitute a Valine amino acid to Methionine at position 291 (Beutler et al., 1991).

G6PD Viangchan belongs to WHO Class II which only produces 10 % of the total enzyme activity (severe G6PD deficiency). The same 871 G>A mutation was also found in G6PD Jammu which is originated from India (Beutler et al., 1991). The only difference between the two variants is nucleotide polymorphism at position 311, where it was C in G6PD Jammu and T in G6PD Viangchan. G6PD Viangchan was found to be the most common variant in Laotians (Iwai et al., 2001, Hsia et al., 1993), Malaysian Malays (Ainoon et al., 2003, Yusoff et al., 2004), Cambodians (Matsuoka et al., 2005, Louicharoen and Nuchprayoon, 2005) and Vietnamese (Matsuoka et al., 2007).

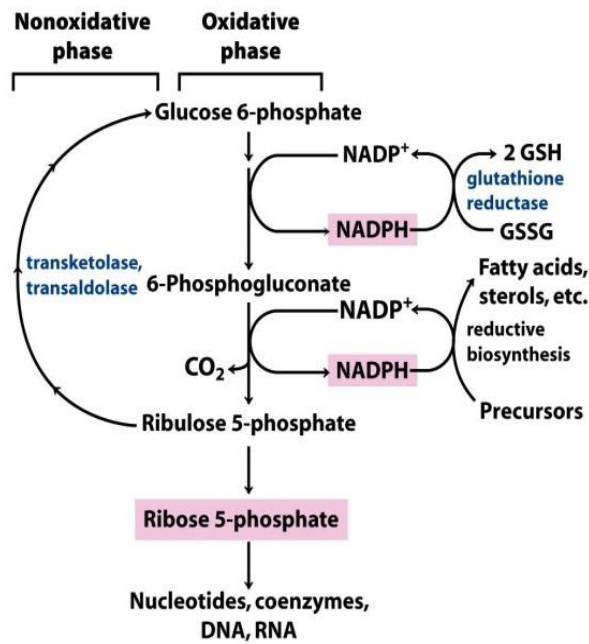


Figure 2.8: Non-oxidative and oxidative pathways of PPP and the paths involved in NADP and GSH production (Nelson et al., 2008). Image also shows the reduction of GSH upon the reduction of NADP. G6PD gene pathway is very crucial in maintaining cellular redox.

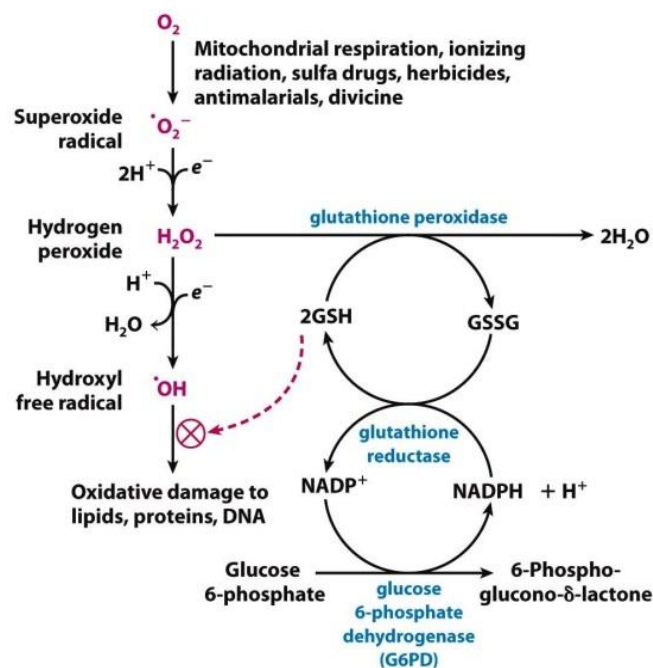


Figure 2.9: Oxygen species are generated by cellular processes such as respiration and redox enzymes. Oxygen species is converted to hydrogen peroxide and it is detoxified through a metabolic pathway. Catalase and peroxidases then convert hydrogen peroxide to water (Nelson et al., 2008).

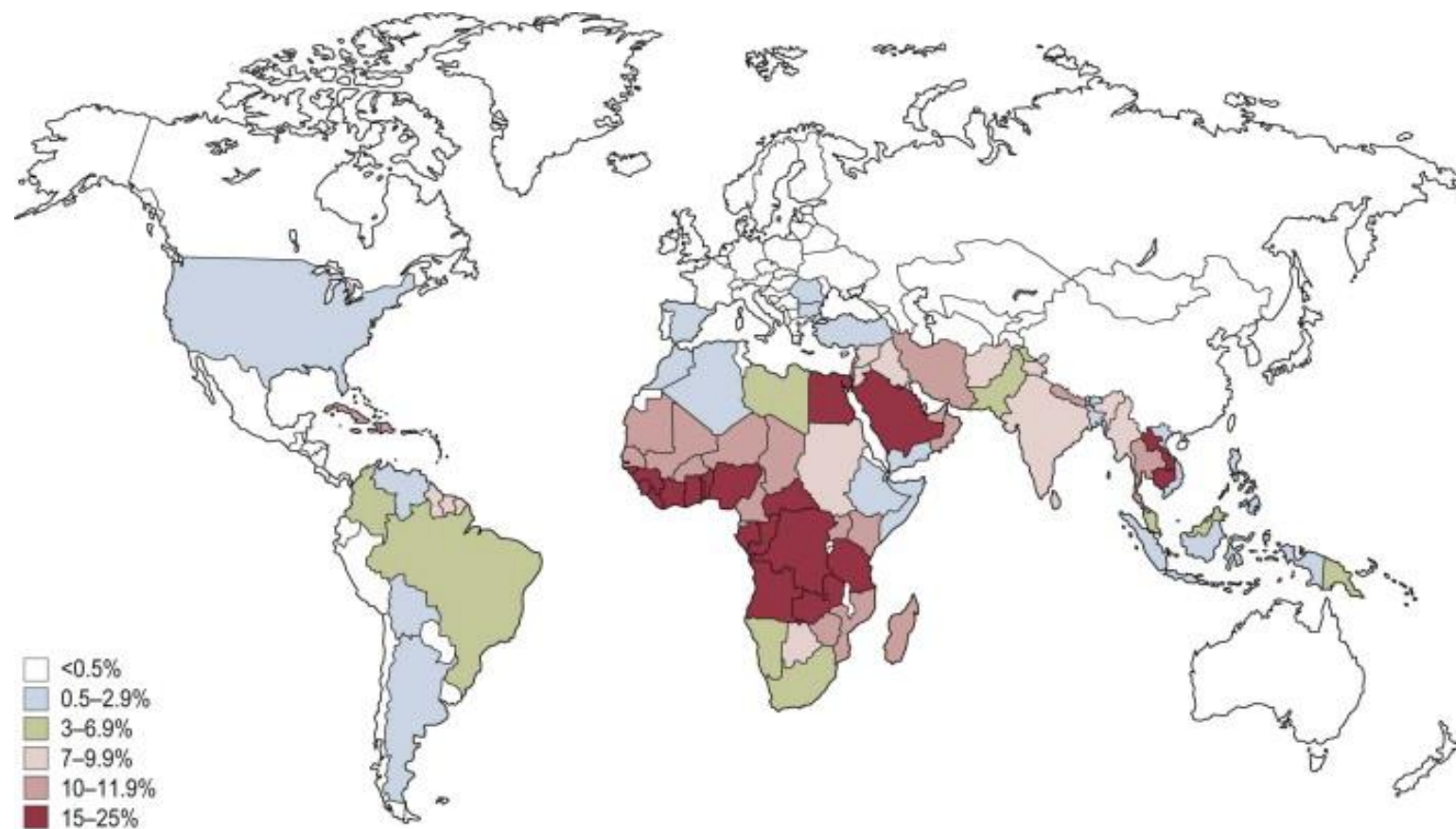


Figure 2.10: Frequency of G6PD deficiency worldwide. The frequency is higher in countries with tropical weather. The highest frequency is found across African and South East Asian countries. (Cappellini and Fiorelli, 2008)

2.3.6 Clinical Presentation

Acute haemolysis anaemia

Patients with G6PD deficiency commonly appears are asymptomatic unless triggered with oxidants (drug or fava bean) (Figure 2.11) or infection (Hosnut et al., 2008). The most usual symptom in patients is haemolytic anaemia but normally periodic. They present with anaemia, increased red cell destruction or in alteration in blood morphology (Corash et al., 1980, Brewer et al., 1961) A paper from Dern 1954 shows that subjects (African origin) with G6PD A- shows acute haemolytic following the administration of primaquine at day 2 to 4 (Fernando et al., 2011). Figure 2.12 shows the appearance of RBC that undergoes haemolytic anaemia.

Sudden onset of jaundice, pallor and dark urine, with or without abdominal and back pain was observed. A same pattern of haemolysis was witnessed among Caucasians and Asian (Bouma et al., 1995). Mediterranean B⁻ variant, on the other hand, is vulnerable to life-threatening haemolysis when treated with primaquine (Beutler 1991). There are also chemical products that commonly causes haemolysis which includes naphthalene (moth balls) (Figure 2.13), aniline dyes, and henna compounds used for hair dyes and tattoo (Figure 2.14) (Raupp et al., 2001).

Another common factor of haemolysis is an infection (Shannon and Buchanan, 1982, Burka et al., 1966). Although the degree of haemolysis is usually mild, massive intravascular haemolysis can cause acute renal failure. Several infectious agents have been described including salmonella (Chan et al., 1971, Hersko and Vardy, 1967, Constantopoulos et al., 1973), *E coli* (Burka et al., 1966), beta-haemolytic streptococci (Mengel et al., 1967), rickettsia (Mengel et al., 1967) and viral hepatitis (Phillips and Silvers, 1969, Salen et al., 1966).

Neonatal jaundice/hyperbilirubinemia

G6PD deficiency is one of the major risk factors for severe neonatal jaundice (Kaplan and Hammerman, 2009). Jaundice can be very severe in some G6PD-deficient babies, especially in association with prematurity, infection, and/or environmental factors (such as naphthalene-camphor balls used in babies' bedding and clothing). Figure 2.15 shows deposition of bilirubin in the basal ganglia (yellow deposited) in kernicterus patient. G6PD related neonatal jaundice rarely present at birth but shows clinical onset is between day 2 and 3 (Kaplan and Hammerman, 2004). A huge study comprised a total of five cohorts with 21 585 participants in the year 2015 shows that 3.92 % of G6PD deficiency neonates have a relative risk of hyperbilirubinemia (Liu et al., 2015). Another study from USA Kernicterus Registry from 1992 to 2004 revealed that 30% of the kernicterus cases are associated with G6PD deficiency (Johnson et al., 2009). Neonatal jaundice with G6PD deficiency can result in kernicterus or bilirubin encephalopathy and permanent neurologic damage (Weng and Chiu, 2010, Dhillon et al., 2003, Valaes, 1994) if not managed poorly. Figure 2.16 shows clinical presentation of babies with neonatal jaundice.

Chronic non-spherocytic haemolytic anaemia (CNSHA)

CNSHA is the most severe form of G6PD-deficiency (Costa et al., 2000). CNSHA classified under G6PD deficiency Class I which produced less than 10 % of normal G6PD enzyme production. As in 2000, 61 G6PD molecular variants associated with CNSHA have been identified but not all of them cause a severe reduction in the enzyme activity (Fiorelli et al., 2000). Many patients with CNSHA caused by G6PD deficiency have a history of severe neonatal jaundice, chronic anaemia often requiring a blood transfusion. Patients also present with lifelong chronic haemolytic anaemia

accompanied by episodes of acute crisis triggered by infections or the ingestion of certain drugs (Francis et al., 2013).

2.3.7 Management

Avoiding any of the triggers is a common management to avoid haemolysis in G6PD deficiency. In a very rare case, when anaemia is severe, a blood transfusion may be necessary. Supplements such as folic acid and iron might possibly be useful in haemolysis too. Infants with prolonged neonatal jaundice which is caused by G6PD deficiency will receive phototherapy with blue light and perhaps an exchange transfusion to prevent kernicterus. Haemolysis that is caused by infection, the cause of the infection is normally treated first. Antibody prescription will be carried out to cure the infection which will eventually subside the triggered haemolysis.

Therefore, in this experiment, we would like to propose gene therapy based treatment to cure the deficiency. Gene therapy treatment has been widely used for many genetic diseases as well. Gene therapy treatment provides both transient and stable gene correction. Please refer the next chapter for a detailed explanation of gene therapy.



Figure 2.11: Fava bean consumption disrupts cellular function in G6PD deficient and causes AHA.

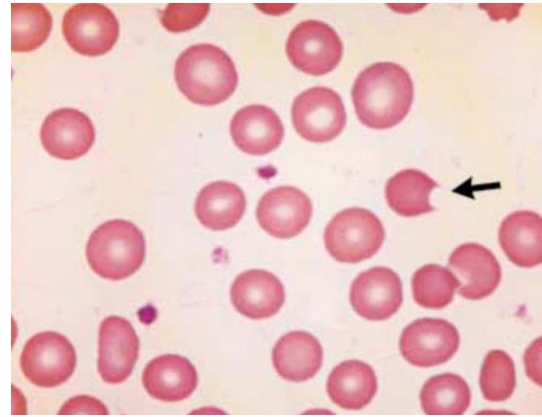


Figure 2.12: Acute haemolysis in glucose-6-phosphate dehydrogenase (G6PD) deficiency (Bain, 2005).



Figure 2.13: Mothball contains compound called naphthalene that can trigger hemolysis.



Figure 2.14: Henna contains lawsone, a similar oxidative property as mothball that can cause hemolysis in G6PD deficient patients.



Figure 2.15: Postmortem specimens showing bilirubin deposition in the basal ganglia (yellow deposited) in kernicterus patient (Christensen et al., 2013).



Figure 2.16: Clinical presentation of babies with neonatal jaundice. Baby's skin and sclerae appear to be slightly yellow.